

Effect of the Tumor Vascular-Damaging Agent, ZD6126, on the Radioresponse of U87 Glioblastoma

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ABSTRACT

Purpose: The effect of ZD6126 on tumor oxygen tension and tumor growth delay in combination with ionizing radiation was examined in the human U87 glioblastoma tumor model. Resistance to ZD6126 treatment was investigated with the nitric oxide synthase inhibitor, L-N^G-nitro-arginine methyl ester (hydrochloride; L-NAME/active form, L-NNA).

Methods: U87 human xenografts were grown in athymic nude mice. ZD6126 was given with or without L-NNA. Tumor oxygen tension was measured using the Oxford Oxylite (Oxford, England) fiberoptic probe system. Tumor volume was determined by direct measurement with calipers and calculated by the formula [(smallest diameter)² × widest diameter]/2.

Results: Multiple doses of ZD6126 treatment (three doses) had a significant effect on tumor growth delay, reducing the average daily tumor growth rate from 29% to 16%. When given 1 hour before radiation, ZD6126 caused an acute increase in hypoxia in U87 tumors, and reduced tumor growth delay compared with that of radiation alone. The combination of ZD6126 given after radiation, either as a single dose or in multiple doses, had greater or similar antitumor activity compared with radiation alone. Twenty-four hours after administration, a single dose of ZD6126 induced little (10 ± 8%) necrosis in U87 xenografts. L-NNA, when given in

combination with ZD6126, significantly enhanced the effectiveness of ZD6126 in inducing tumor necrosis.

Conclusions: Our observation that ZD6126-induced tumor hypoxia can decrease radiation response when ZD6126 is given prior to radiation indicates the importance of scheduling. Our findings suggest that the optimal therapeutic benefit of ZD6126 plus radiation in human glioblastoma may require multiple dosing in combination with a nitric oxide synthase inhibitor, to be scheduled following radiotherapy.

INTRODUCTION

Continued growth and survival of solid tumors depend on a competent and expanding tumor vascular supply. Tumors without an adequate blood supply become necrotic or apoptotic and fail to grow beyond 2 to 3 mm in size (1). Recently, a new class of antivascular drugs (antiangiogenic and tumor vascular-damaging/targeting agents) has been developed which target the genetically stable normal endothelial cells of tumor blood vessels rather than the genetically unstable cells of the tumor itself (2, 3). Antiangiogenic therapy aims to inhibit the formation of new blood vessels within the tumor, whereas vascular-damaging agents target preexisting tumor blood vessels, causing rapid and catastrophic shutdown of vascular function and secondary tumor cell death (4). Currently, two major classes of low molecular weight drugs that can elicit irreversible vascular shutdown are in clinical development: the tubulin-binding microtubule-destabilizing agents (5), such as ZD6126, and drugs related to flavone acetic acid (6).

The novel vascular-damaging agent, ZD6126 (*N*-acetylcolchicol-*O*-phosphate), is a phosphate prodrug, that is rapidly converted *in vivo* to the active microtubule-destabilizing moiety, ZD6126 phenol. Selective effects of microtubule-destabilizing agents on tumor blood vessels are thought to arise because immature endothelial cells present in tumor vasculature have a poorly developed actin cytoskeleton, relying instead on a microtubule network to maintain cell shape and function (4). Following exposure to the drug, inhibition of tubulin polymerization and microtubule destabilization induce a rapid change in morphology of immature endothelial cells, resulting in a loss of vessel function associated with vessel occlusion. This blocks tumor blood supply and induces subsequent massive tumor necrosis (7, 8) due to oxygen and nutrient starvation. Previous studies with ZD6126, and other vascular-damaging agents, in a variety of tumor models, have shown that despite massive central necrosis, a viable rim of tumor cells survives at the tumor periphery. One hypothesis is that this viable rim survives treatment because blood vessels in the surrounding normal tissue, which remain unaffected by the drug, can provide oxygen and nutrients (9–12). Alternatively, damage to tumor vasculature at the periphery of the tumor may not be permanent, and recovery of vessel function in this region of the tumor may give rise to the viable rim (8). Nevertheless, this viable rim undermines the

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effectiveness of vascular-damaging agents because tumors can regrow quickly from these residual tumor cells. However, combining ZD6126 with radiation therapy has been reported to result in greater tumor cell killing and tumor growth delay, compared with single agent therapy, presumably because the rim of tumor tissue that survives ZD6126 treatment is still oxygenated and therefore presents an ideal target for ionizing radiation, a therapeutic modality which is most effective in well-oxygenated areas of tumors. Accordingly, several studies have reported an increased antitumor effect with the complementary approach of selectively damaging tumor vasculature combined with radiation treatment (11–13).

The optimal schedule for combining ionizing radiotherapy with vascular-damaging agents has not yet been determined. Scheduling was found to be an important factor in the efficacy of ZD6126 when combined with radiation (12). ZD6126 increased tumor cell killing in KHT murine sarcoma when given 24 hours prior to radiation or 1 hour or more after radiation, but was found not to be as effective if given 1 hour prior to radiation. It was suggested that blood flow needs to be reestablished in the remaining viable tissue in order to obtain maximum radiosensitization of the tumor.

The aim of this study was to determine the effect of ZD6126 on tumor oxygenation and tumor growth delay in the highly aggressive, highly angiogenic U87 glioblastoma tumor model, which is very resistant to various forms of cancer therapy. The goal of this study was to enhance U87 tumor response to radiation and to determine optimal scheduling of ZD6126 administration in relation to radiation therapy. Compared with other models reported in the literature (7, 14), U87 was shown to be relatively resistant to induction of tumor necrosis following ZD6126 treatment. Therefore, additional experiments were done to examine the role of nitric oxide (NO) in U87 tumors, because NO has been reported to be an important factor in resistance of certain tumor models to combretastatin A4, another microtubule-destabilizing vascular-damaging anticancer agent (15).

MATERIALS AND METHODS

Animal and Tumor Model

U87 glioblastoma cells (American Type Culture Collection, Manassas, VA) were maintained in alpha MEM (Sigma, St. Louis, MO) with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). U87 cell suspension was injected s.c. into the right hind limb (5×10^5 cells in 100 μ L PBS) of athymic NCR NUM mice (Taconic Farms, Germantown, NY). Mice were not pretreated prior to tumor implantation.

U87 tumors were allowed to grow for approximately 14 to 18 days until reaching an approximate volume of 150 to 400 mm³, before treatment. Tumor volume was determined by direct measurement with calipers and calculated by the formula [(smallest diameter)² \times widest diameter]/2]. Tumors were not allowed to grow > 2,000 mm³ in accordance with Institutional Animal Care and Use Committee regulations.

Statistics

Mixed-effects linear regression was used to model the base-10 logarithm of tumor volume as a function of time and treatment. This approach fits a "random" curve to each animal's

data and then statistically "averages" these curves within each treatment group to obtain the overall "fixed effect" for each group. This method appropriately handles unbalanced data (i.e., different number of measurements for different animals) and takes into account the correlation of each animal's measurements over time. The analyses were conducted in SAS 8.2 (SAS Institute, Inc., Cary, NC, 1999–2001).

Drug Treatment

ZD6126 (AstraZeneca, Macclesfield, United Kingdom) was dissolved in PBS containing 0.5% sodium carbonate. ZD6126 was given i.p. at concentrations indicated in RESULTS. L-NNA was obtained from Cayman Chemicals (Ann Arbor, MI).

Irradiation

Irradiations were done on anesthetized mice using an X-ray machine (Pantak, East Haven, CT) operating at 250 kV, 10 mA, with a 2-mm aluminum filtration. The effective photon energy was \approx 90 keV. Mice were anesthetized with a combination of ketamine and acepromazine at a concentration of 75 and 0.35 mg/kg, respectively. Each mouse was confined in a lead casing with its tumor-bearing leg extended through an opening on the side to allow the tumor to be irradiated locally.

Tumor Oxygen Measurements

Tumor oxygen was determined using the Oxford Oxylite fiberoptic probe (Oxford, England). The detection system is based on blue light excitation of ruthenium pigment, excitation is quenched by oxygen. Tumor volume chosen for this study ranged between 150 and 250 mm³. Oxygen measurements were done on anesthetized mice; body temperature was maintained at approximately 37°C with a heating pad. A 25-gauge needle was used to puncture the tumor capsule to facilitate insertion of the fiberoptic probe. The probe was guided into the approximate center of the tumor at about 3 to 4 mm in depth. An initial oxygen baseline measurement was done for approximately 50 minutes. Drug or drug vehicle was then injected i.p. and followed by pO₂ monitoring for an additional 1 hour. Anesthesia was given every 30 minutes at half the dose to keep the animal anesthetized. Both temperature and pO₂ was simultaneously measured and recorded by the Oxylite software program.

In vivo Tumor Necrosis

U87 tumors were allowed to reach 400 mm³, at which time mice received a single i.p. injection of L-NNA at 20 mg/kg immediately followed by a single i.p. injection of ZD6126 at 200 mg/kg. Tumors were collected 24 hours later for fixation and staining with H&E. The area of necrosis was evaluated by image analysis and expressed as the percentage of the total tumor area.

Analysis of NO Levels in U87 Tumor Cells in Culture

A colorimetric nonenzymatic assay for measuring total NO (nitrate plus nitrite, Oxford Biomedical Research) was used. This kit employs metallic cadmium for quantitative conversion of nitrate to nitrite prior to quantification of nitrate using Griess reagent. U87 cells were plated in triplicate into 96-well plates and assayed for NO production 7 days after plating.

Analysis of VEGF Levels in U87 Tumor Cells in Culture

U87 cells were plated into six-well plates and incubated for 2 days. VEGF was assayed from culture supernatants using a commercially available human VEGF immunoassay kit (Quantikine).

RESULTS

Effect of ZD6126 on Tumor Oxygenation in U87 Tumor Xenografts

Tumor oxygen tension was examined in U87 xenografts following ZD6126 treatment (150 mg/kg). The effect of ZD6126 administration on tumor oxygen tension in a representative U87 glioblastoma xenograft implanted in the hind limb of a nude mouse is shown in Fig. 1. A summary of oxygen tension measurements obtained from pO_2 tracings from all experimental animals is shown in Fig. 2. The average observed pretreatment tumor pO_2 was 5 with a SE of ± 4 mm Hg in animals breathing air, which was reduced to ≤ 0.5 mm Hg following treatment with ZD6126. Because the pretreatment tumor pO_2 levels were relatively low in air-breathing animals, we also examined the effect of ZD6126 in animals breathing 100% oxygen. In this case, the pretreatment tumor pO_2 was increased to 60 ± 29 mm Hg, which declined to an average pO_2 of 6 ± 3 mm Hg within 46 minutes of ZD6126 administration. Thus, one dose of ZD6126 acutely reduced tumor oxygen tension levels in the U87 tumor model, causing severe hypoxia throughout the period of monitoring (1 hour). The effect was selective for tumor pO_2 , with no evidence of ZD6126-induced hypoxia in nontumor s.c. tissue.

Induction of Tumor Necrosis by ZD6126 in the U87 Xenograft Model

Previously, ZD6126 was reported to induce massive necrosis (7) in a range of histologically diverse human tumor xenografts. Single doses of 100 and 200 mg/kg ZD6126 induced necrosis in > 70% or > 80% of the tumor mass, respectively, in human tumor

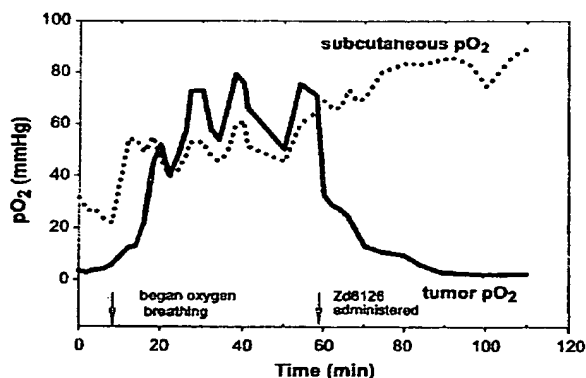


Fig. 1 Effect of ZD6126 administration on tumor oxygen tension in a representative U87 glioblastoma xenograft. pO_2 was continuously monitored up to 2 hours with an Oxylite fiberoptic probe inserted at a depth of approximately 2 to 3 mm into the tumor (average tumor size: length, 6.6 ± 1 mm; width, 7.7 ± 0.15 mm). Readings were obtained with or without oxygen breathing over a period of 2 hours.

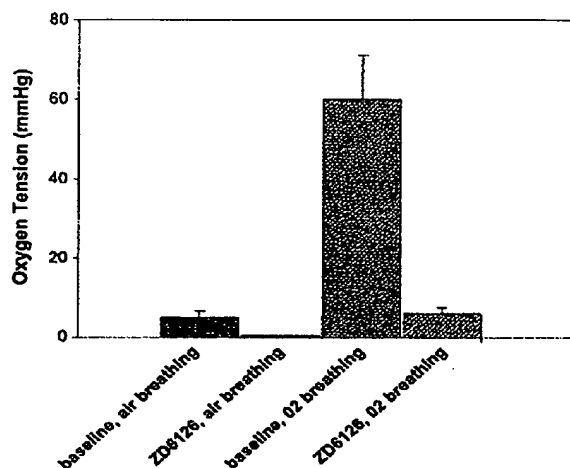


Fig. 2 Average oxygen tension in U87 glioblastoma after administration of ZD6126. pO_2 was continuously monitored up to 2 hours with an Oxylite fiberoptic probe inserted at a depth of approximately 2 to 3 mm into the tumor. Readings were obtained with and without oxygen breathing over a period of 2 hours. Mean pO_2 values collected during the period of monitoring for each treatment as indicated in Fig. 1 (\pm SE for five to eight tumors; tumor volume, 70 ± 6 mm³).

xenografts (7). Compared with previous tumor models, U87 was relatively insensitive to induction of necrosis, with single doses of ZD6126 (150 mg/kg) inducing $10 \pm 8\%$ tumor necrosis compared with control values of $2 \pm 4\%$ (Fig. 3; Table 1).

Effect of a Single Dose of ZD6126 on U87 Tumor Growth Inhibition by a Single Dose of Radiation

The main goal of this study was to evaluate the effect of combined treatment with ZD6126 and radiation on U87 tumor growth. Four sets of experiments were conducted (see Table 2) examining single doses (experiments 1 and 2) or multiple doses (experiments 3 and 4).

In experiments 1 and 2 (Tables 3 and 4), tumor growth delay was examined in large ($250\text{--}400$ mm³) and small U87 tumors ($150\text{--}250$ mm³) following administration of a single dose of ZD6126 (150 mg/kg) 1 hour before or 1 hour after a single dose of radiation (10 Gy on day 0). In the control groups, U87 tumors grew by an average of 28% to 29% a day, corresponding to a tumor doubling time of 2.7 to 2.8 days. Compared with controls, radiotherapy (10 Gy) alone significantly inhibited tumor growth, decreasing tumor growth rate per day to an average 12% ($P < 0.001$) or 14% ($P < 0.001$) in large and small U87 tumors, respectively. A single dose of ZD6126 (150 mg/kg) did not have a significant effect on U87 tumor growth in either large or small tumors (Tables 3 and 4). In addition, once-weekly doses of ZD6126 (150 mg/kg) did not affect U87 tumor growth (Table 3). Combining ZD6126 (150 mg/kg) either 1 hour before, or 1 hour after radiotherapy (10 Gy) did not result in greater antitumor activity compared with radiotherapy alone. Although ZD6126 given 1 hour following radiation induced a tumor growth delay that was comparable to radiation alone, the groups receiving ZD6126 1 hour prior to radiation treatment had a trend towards faster average daily tumor growth rates than groups receiving radiation alone. This effect was seen both in large

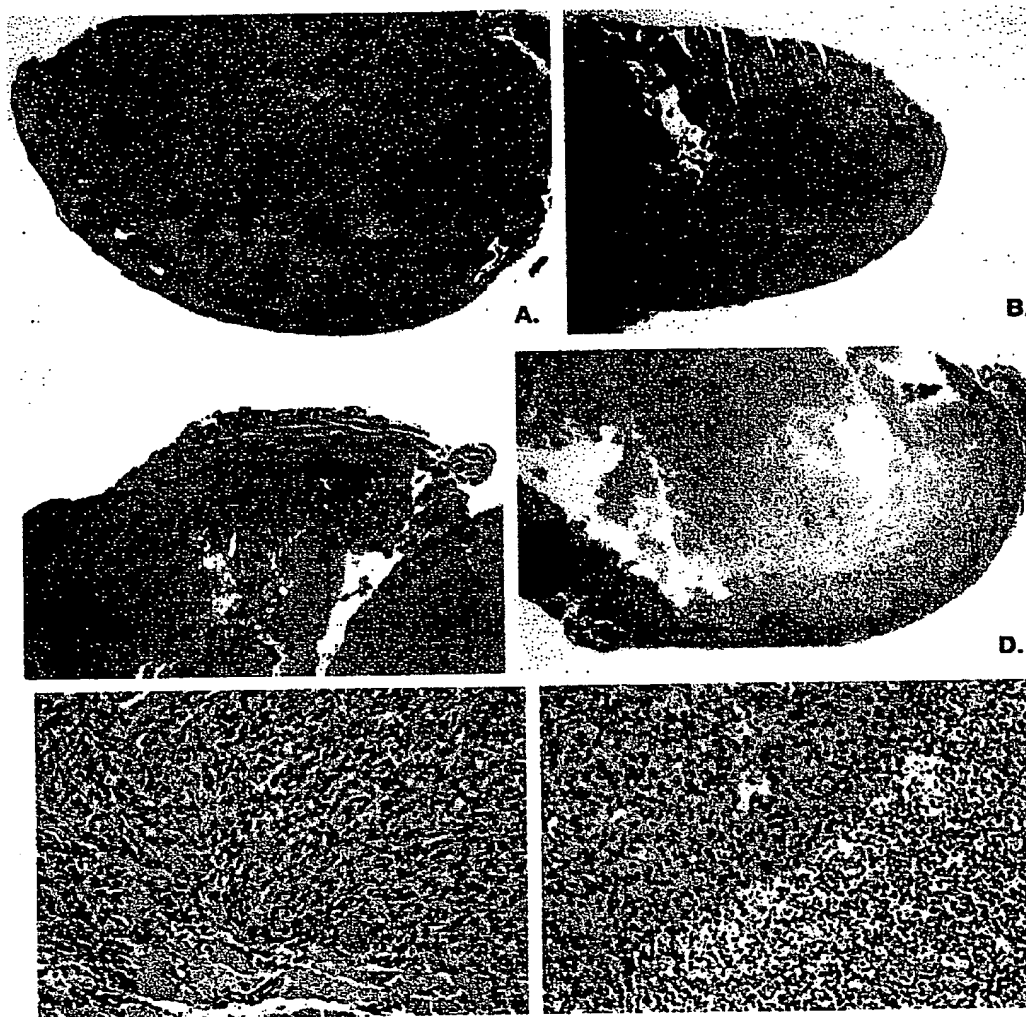


Fig. 3 H&E stained sections of U87 glioblastoma xenografts. *A*, control (untreated) tumor showing no necrosis. *B*, tumor from animal 24 hours after administration of ZD6126 (200 mg/kg) showing 10% necrosis. *C*, tumor from animal 24 hours after administration of L-NNA (20 mg/kg) showing 15% necrosis. *D*, tumor from animal 24 hours after administration of ZD6126 (200 mg/kg) immediately followed by L-NNA (20 mg/kg). Note the excessive central tumor necrosis (lighter staining; 95% of total area) in contrast to viable tissue in the periphery. *E*, control tumor at higher magnification. *F*, higher magnification of tumor 24 hours after administration of ZD6126 and L-NNA. Note evidence of necrosis in lighter staining area showing cell shrinkage, disruption, and fragmentation. (*A-D*, 20 \times magnification; *E* and *F*, 200 \times magnification).

tumors, where the difference was statistically significant (Table 3: 19% versus 12%, $P = 0.014$) and in small tumors, although this did not achieve statistical significance (Table 4: 20% versus 14%, $P = 0.068$).

Table 1 Induction of tumor necrosis by ZD6126 and/or L-NNA in U87 glioblastoma tumor

Group	Necrosis (% total area \pm SD)
Control ($n = 5$)	2 \pm 4
ZD6126 ($n = 4$)	10 \pm 8
L-NNA ($n = 5$)	24 \pm 14
ZD6126 + L-NNA ($n = 4$)	92 \pm 3

Effect of Multiple Doses of ZD6126 on U87 Tumor Growth Inhibition by Fractionated Doses of Radiation

In experiments 3 and 4 (Fig. 4A and B; Tables 5 and 6) the effect of ZD6126 on U87 tumor growth delay was examined using multiple doses of drug with fractionated radiotherapy. In these experiments, small U87 tumors (150-250 mm³) were treated on days 0, 2, and 4 with either high dose fractionated radiotherapy (7.5 Gy \times 3) or lower dose (5 Gy \times 3) plus ZD6126 (150 mg/kg) given either 1 hour before or 1 hour after each dose of radiation.

Control U87 tumors grew by an average of 28% to 29% a day, corresponding to a tumor doubling time of 2.7 to 2.8 days.

Table 2 Experimental design of the four experiments studying the effect of ZD6126 and/or radiation on U87 tumor growth

Tumor treatment	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Groups	(250-450 mm ³)*	(150-250 mm ³)*	(150-250 mm ³)*	(150-250 mm ³)*
Control	No treatment	No treatment	No treatment	No treatment
ZD6126 alone	150 mg/kg	150 mg/kg	3 × 150 mg/kg	3 × 150 mg/kg
Radiation alone	10 Gy	10 Gy	3 × 7.5 Gy	3 × 5.0 Gy
ZD6126 before radiation	150 mg/kg, 1 h before radiation (10 Gy)	150 mg/kg, 1 h before radiation (10 Gy)	150 mg/kg, 1 h before each 7.5 Gy fraction	150 mg/kg, 1 h before each 5.0 Gy fraction
ZD6126 after radiation	150 mg/kg, 1 h after radiation (10 Gy)	150 mg/kg, 1 h after radiation (10 Gy)	150 mg/kg, 1 h after each 7.5 Gy fraction	150 mg/kg, 1 h after each 5.0 Gy fraction
ZD6126 once weekly for 4 weeks	150 mg/kg	150 mg/kg	None	None

NOTE. n = 7 (experiments 1 and 2); n = 10 (experiments 3 and 4).

*Tumor volume at start of treatment.

consistent with experiments 1 and 2. Fractionated radiotherapy alone significantly inhibited U87 growth, with average daily tumor growth reduced to 6% ($P < 0.001$ versus control) and 14% ($P < 0.001$ versus control) in the high dose and lower dose fractionated radiotherapy groups, respectively. Compared with controls, single agent ZD6126 (150 mg/kg, days 0, 2, and 4) also significantly inhibited U87 tumor growth, reducing daily growth rate to 16% ($P < 0.001$) and 21% ($P = 0.009$) in experiments 3 and 4, respectively, although magnitude of inhibition did not seem as marked as with radiation treatment. The effectiveness of this schedule of ZD6126 (3 × 150 mg/kg i.p. injections over 5 days) was in contrast to weekly i.p. injections of ZD6126 (150 mg/kg), which was not an effective schedule in the U87 tumor model (see Tables 3 and 4).

Combining ZD6126 1 hour after each dose of fractionated radiotherapy resulted in similar antitumor activity to radiotherapy alone, both in the high dose and lower dose radiotherapy groups (Tables 5 and 6, respectively). Although there was a possible trend towards increased tumor doubling time with this combination, there was no statistical significance (Fig. 4; Tables 5 and 6).

In contrast, combining ZD6126 1 hour before each dose of radiotherapy seemed less effective antitumor therapy than radiotherapy alone, in particular, in combination with high dose fractionated radiotherapy. This combination produced average daily tumor growth rates of 12%, significantly faster than the growth rate of 6% under fractionated radiotherapy alone ($P < 0.001$; Fig. 4; Table 5). No statistically significant difference was seen when ZD6126 was combined 1 hour prior to lower dose fractionated radiotherapy ($P = 0.25$; Table 5). Both combination schedules of ZD6126 with fractionated radiotherapy produced

significantly lower tumor growth rates than untreated controls (Tables 5 and 6).

Effect of L-NNA on the Induction of Tumor Necrosis by ZD6126

Despite inducing hypoxia in the U87 human tumor xenograft, a single injection of ZD6126 induced only modest levels of tumor necrosis (2-18%; Fig. 3B; Table 1) rather than the massive tumor necrosis reported for other human tumor xenograft models (7). Because inhibitors of NO synthase (NOS) have been reported to overcome resistance to combretastatin A4 phosphate in the SaS murine sarcoma tumor model (16), the effect of L-NNA with and without ZD6126 was examined. Twenty-four hours after dosing, a single dose of L-NNA alone produced 10% to 38% necrosis (Fig. 3C; Table 1). When ZD6126 was combined with L-NNA, the percentage of necrosis was increased to 89% to 95% (Fig. 3D; Table 1). The extent and pattern of necrosis was characteristic of the pattern seen with vascular-damaging agents in previous studies, i.e., a massive necrosis at the center with a viable rim at the periphery (see Fig. 3D).

Effect of L-NNA on VEGF Secretion and NO Levels in U87 Glioblastoma Cells in Culture

VEGF protein secretion was measured in supernatants from U87 cells maintained in culture for 48 hours. The VEGF level was ~25,000 pg/mL/(1 × 10⁶) tumor cells and L-NNA (1 mmol) did not inhibit VEGF secretion. In addition, we determined the basal level of NO in U87 cells in culture for 6 days to be 29 ± 6 μmol/L, using the Griess reaction. NO levels in the presence of L-NNA could not be accurately determined due to high

Table 3 Experiment 1: estimates for the geometric mean tumor volume (in mm³) over time, the mean percent increase per day, and the doubling time (in days), by treatment group

Treatment	Time (days)							%Δ (95% CI)	T _{2,Δ}
	0	1	2	3	5	7	10		
Control	326	418	536	687	1,130	1,857	*	28 (23, 33)	2.8
ZD	360	446	554	687	1,057	1,627	*	24 (19, 29)	3.2
ZD6126 (4 ×)	321	411	527	676	1,110	1,825	*	28 (23, 34)	2.8
Radiation	378	423	473	529	662	829	1,160	12 (8, 16)	6.2
ZD6126+Radiation	384	458	547	652	929	1,324	*	19 (15, 24)	3.9
Radiation+ZD6126	393	458	534	623	848	1,155	1,833	17 (13, 21)	4.5

NOTE. %Δ, rate of increase of tumor volume (average % daily increase); T_{2,Δ}, average doubling time for tumor volume (in days).* Predicted geometric mean > 2,000 mm³ (beyond data range).

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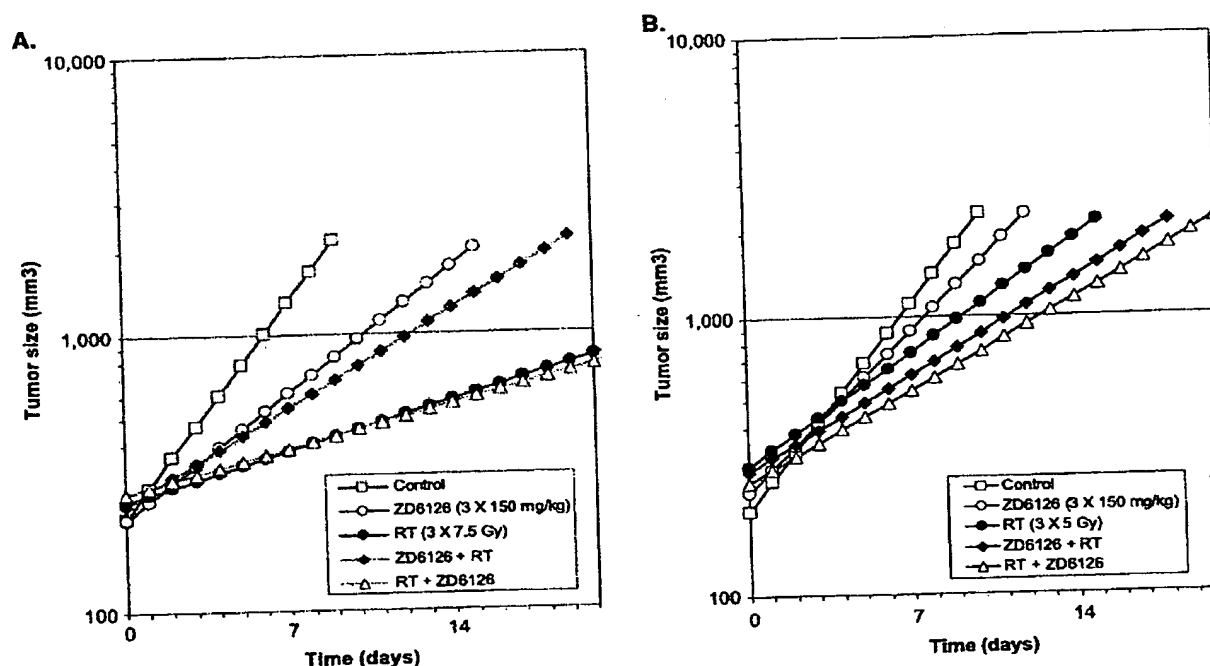


Fig. 4 Tumor growth delay in U87 glioblastoma xenografts. A, tumor size ranged between 150 and 250 mm³ at start of treatment on day 0. Irradiation was given in three fractions of 7.5 Gy each, on days 0, 2, and 4. ZD6126 (150 mg/kg) was given before or after each fraction. *n* = 10 animals per group. B, tumor size ranged between 150 and 250 mm³ at start of treatment (day 0). Irradiation was given in three fractions of 5.0 Gy each, on days 0, 2, and 4. ZD6126 (150 mg/kg) was given before or after each fraction. *n* = 10 animals per group.

background values of NO measured by the Griess reaction in the presence of 1 mmol L-NNA (data not shown).

Effect of L-NNA on the Antitumor effects of ZD6126 in the U87 Tumor Xenograft Model

Preliminary experiments to determine the effect of L-NNA on the antitumor activity of ZD6126 were done. A single dose of L-NNA (20 mg/kg) and ZD6126 (200 mg/kg) following a single dose of 10 Gy showed no significant additional benefit over ZD6126 and radiation without inhibitor (*P* = 0.135). Multiple dosing schedules with L-NNA are currently being pursued.

DISCUSSION

In the present study of U87 human glioblastoma xenografts grown in nude mice, a single dose of ZD6126 (150 mg/kg) induced only modest levels of tumor necrosis. This is in contrast

to previous studies where similar dosing of ZD6126 induced massive central necrosis in a range of histologically diverse human tumor xenografts models, including LoVo, Has5, Calu-6, HT29, PC-3, SKOV-3, and MDA-MB-231 (7, 14). A modest induction of tumor necrosis in the U87 tumor model was apparent despite a marked, ZD6126-induced, tumor selective hypoxia that lasted for at least 1 hour (the period of monitoring).

Because a single dose of ZD6126 produced acute hypoxia, and only modest levels of tumor necrosis, it was considered that ZD6126 might cause tumor hypoxia by a direct effect on tumor metabolism, i.e., by causing an increase in tumor cell oxygen consumption. However, ZD6126 was found not to affect oxygen consumption in an *in vitro* assay monitoring oxygen consumption in U87 cells in suspension (data not shown). Therefore, ZD6126-induced tumor hypoxia seems to be the result of a direct effect on tumor vasculature.

Table 4 Experiment 2: estimates for the geometric mean tumor volume (in mm³) over time, the mean percent increase per day, and the doubling time (in days), by treatment group

Treatment	Time (days)							%Δ (95% CI)	T ₂
	0	1	2	3	5	7	10		
Control	188	243	314	405	675	1,125	2,420	29 (25, 33)	2.7
ZD6126	171	219	280	358	587	962	2,017	28 (24, 32)	2.8
ZD6126 (4x)	188	239	303	384	616	990	2,017	27 (23, 31)	2.9
Radiation	251	286	325	369	477	617	908	14 (10, 17)	5.4
ZD6126+Radiation	217	260	311	371	531	759	1,298	20 (16, 23)	3.9
Radiation+ZD6126	219	257	301	352	483	662	1,064	17 (14, 21)	4.4

NOTE. %Δ, rate of increase of tumor volume (average % daily increase); T₂, average doubling time for tumor volume (in days).

Table 5 Experiment 3: estimates for the geometric mean tumor volume (in mm³) over time, the mean percent increase per day, and the doubling time (in days), by treatment group

Treatment	Time (days)								%Δ (95% CI)	T _{2x}
	0	1	2	3	5	7	10	14		
Control	217	280	362	467	779	1,299	*	*	29 (24, 34)	2.7
ZD6126	216	251	291	338	455	613	959	1,742	16 (13, 20)	4.6
Radiation	250	265	281	298	336	379	453	575	6 (3, 9)	11.6
ZD6126+Radiation	242	272	305	343	432	544	771	1,225	12 (9, 15)	6.0
Radiation+ZD6126	267	281	296	312	347	385	451	557	5 (3, 8)	13.2

NOTE. %Δ, rate of increase of tumor volume (average % daily increase); T_{2x}, average doubling time for tumor volume (in days).

* Predicted geometric mean > 2,000 mm³ (beyond data range).

The protective effect of ZD6126 when given 1 hour prior to radiation can be explained by ZD6126-induced tumor hypoxia. The group receiving ZD6126 before radiation had substantially faster tumor growth rate than the group receiving radiation alone in three of the four experiments done in this study where radiation doses were highest (i.e., 3 × 7.5 Gy or 10 Gy). In these experiments, a significant difference is seen between giving ZD6126 before radiation versus administering it following radiation. By inducing hypoxia, ZD6126 abrogates the sensitizing effect of oxygen on radiation-induced cell killing. Furthermore, at the lower dose of 3 × 5 Gy where there is less cell killing and hence less of an oxygen enhancement effect, ZD6126 plus radiation had no effect (see Fig. 2B). These observations are in agreement with findings of Siemann and Rojiani (12) who showed that a combination of ZD6126 and radiation increased tumor cell killing in KHT murine sarcoma when given 24 hours prior to radiation or 1 hour or more after radiation, but was found not to be as effective if given 1 hour prior to radiation.

In the present study, although a three-dose ZD6126 treatment (on days 0, 2, and 4) had a significant effect alone, reducing the average daily tumor growth rate for U87 glioblastoma from 28–29% to 16%. ZD6126 given after radiation, either as a single dose or in multiple doses, tended to be comparable to radiation alone. The failure of ZD6126 to enhance radiation response in U87 glioblastoma as opposed to previously shown efficacy with radiation in other solid tumors (12) was puzzling. It is possible that the site of tumor implantation may play a role in tumor response to vascular targeting. It is known that ectopic tumors implanted s.c. in the hind limb differ from orthotopic implants of gliomas with regard to tumor vascularization (17). It is also possible that the data presented in this study with U87 glioblastoma may not be entirely representative of all gliomas with regard to response to vascular targeting. However, the U87 glioblastoma model is representative of most human malignant gliomas in being a

fast growing, highly angiogenic neoplasm. It is logical to assume that the resistance to vascular targeting and radiation exhibited by U87 in this study is related to the highly angiogenic nature of this tumor, in light of experimental evidence suggesting that antiangiogenic compounds enhance the antitumor effects of ionizing radiation in a variety of glioma models (18, 19).

Although the precise mechanisms of glioma-induced angiogenesis are not clear, certain factors are known to play a role such as stimulation of neovascularization by the VEGF/VEGF receptor signaling pathway and protection of endothelial cell death by tumor cell-induced cytokines such as VEGF. In addition, NO is a major signaling molecule, having several effects on tumor vasculature which may protect against tumor vascular damage (16). These effects include: maintenance of blood flow by a potent vasodilatory effect; removal of ZD6126-induced damaging oxygen species, in particular, superoxide; suppression of inflammatory neutrophil aggregation after endothelial damage (20); protection of endothelial cells under low oxygen conditions (21); and stimulation of neovascularization by NO-induced signaling pathways (22). In this regard, it has been shown that NO in conjunction with hypoxia can induce VEGF gene transcription in human glioblastoma (23, 24). VEGF, in turn can stimulate endothelial and inducible NOS expression in vascular endothelial cells *in vitro* (25).

It was hypothesized that the resistance of U87 glioblastoma to combined targeting by ZD6126 and radiation may result, in part, from NO production by U87 cells. This hypothesis was based on previous studies in rat tumors indicating that NO production offers some protection from the vascular targeting agent, combretastatin A4 phosphate (15, 16, 20). In particular, the round-cell sarcoma, SaS, in CBA mice has been reported to be resistant to combretastatin A4 phosphate and to have a high rate of NO production (20). Administration of the NOS inhibitor, L-NNA, resulted in an enhancement of vascular shutdown.

Table 6 Experiment 4: estimates for the geometric mean tumor volume (in mm³) over time, the mean percent increase per day, and the doubling time (in days), by treatment group

Treatment	Time (days)								%Δ (95% CI)	T _{2x}
	0	1	2	3	5	7	10	14		
Control	202	257	329	420	684	1,116	*	*	28 (24, 31)	2.8
ZD6126	235	284	344	416	609	891	1,577	*	21 (18, 24)	3.6
Radiation	292	334	382	437	571	746	1,115	1,906	14 (12, 17)	5.2
ZD6126+Radiation	279	313	350	393	493	619	870	1,371	12 (9, 15)	6.1
Radiation+ZD6126	255	285	317	353	439	544	753	1,160	11 (9, 14)	6.4

NOTE. %Δ, rate of increase of tumor volume (average % daily increase); T_{2x}, average doubling time for tumor volume (in days).

* Predicted geometric mean > 2,000 mm³ (beyond data range).

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In the present study, administration of L-NNA potentiated the necrosis-inducing effect of ZD6126 in U87 glioblastoma. The level of NO produced in U87 cells in culture in this study indicates that NOS, which exists as three isoforms (neuronal, endothelial, and inducible forms) is being expressed in the tumor cells themselves and contributing to NO synthesis, although it is not clear from this study which NOS isoforms (endothelial, neuronal, or inducible NOS; ref. 26) are involved. The mechanism(s) whereby NO protects U87 glioblastoma against ZD6126-induced vascular damage is not known. In the present study, high levels of VEGF expression were detected in U87 cells in culture ($\sim 25,000$ pg/mL/ 1×10^6 cells); VEGF levels were not diminished in the presence of L-NNA. Therefore, it is not likely that L-NNA is contributing to tumor necrosis *in vivo* by interfering with VEGF secretion from U87 cells directly. Rather, it is more likely that L-NNA, which exhibits selectivity for endothelial and inducible NOS, is inhibiting these isoforms in the endothelial cell compartment of the tumor, leading to reduced endothelial cell NO, reduced blood flow, and vascular necrosis.

There is a great deal of literature suggesting that the levels of NO are important in determining protumor or antitumor growth effects (27). It has been hypothesized that high concentrations of NO have an antitumor effect, whereas at lower concentrations, NO may promote tumor growth (27). This study has shown that ZD6126 can induce tumor hypoxia. Tumor hypoxia is known to stimulate NOS activity. The amount of NO synthesized is not known but it may contribute to protumor growth effects as outlined above. The decrease in NO levels produced by L-NNA may have led to increased tumor cytotoxicity and consequently potentiation of the necrosis-inducing effect of ZD6126 in the U87 tumor model.

In summary, this study has shown that: (a) U87 glioblastoma is highly resistant to a single dose of the vascular targeting agent, ZD6126, alone or in combination with radiotherapy; (b) a single dose of ZD6126 can induce acute tumor hypoxia in U87 glioblastoma; (c) a three-dose ZD6126 treatment (on days 0, 2, and 4) had a significant effect in reducing the average daily U87 glioblastoma growth rate, although it did not enhance tumor response when given in combination with radiotherapy; (d) NO is present in U87 glioblastoma cells in culture and levels can be decreased in the presence of the NOS inhibitor, L-NNA; (e) L-NNA, when given in combination with ZD6126, can significantly enhance the effectiveness of ZD6126 as a necrosis-inducing agent, although a single dose does not lead to a delay in tumor growth. These findings suggest that the optimal therapeutic benefit of ZD6126 plus radiation in human glioblastoma may require multiple dosing in combination with a NOS inhibitor.

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